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Blood Cells, Molecules, and Diseases xx (2006) xxx–xxx

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# Complete loss of iron regulatory proteins 1 and 2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development

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Submitted 10 December 2005; revised 14 December 2005

(Communicated by E. Beutler, M.D., 15 December 2005)

## Abstract

Iron regulatory proteins 1 and 2 (IRPs) are homologous mammalian cytosolic proteins that sense intracellular iron levels and post-transcriptionally regulate expression of ferritin, transferrin receptor, and other iron metabolism proteins. Adult mice with homozygous targeted deletion of IRP2 develop microcytic anemia, elevated red cell protoporphyrin IX levels, high serum ferritin, and adult-onset neurodegeneration. Mice with homozygous deletion of IRP1 develop no overt abnormalities, but mice that lack both copies of IRP2 and one copy of IRP1 develop a more severe anemia and neurodegeneration than mice with deletion of IRP2 alone. Here, we have demonstrated that IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> embryos do not survive gestation, and that although IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> blastocysts can be genotyped and harvested, implanted embryos with the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype are undetectable at embryonic day 6.5 and beyond. Blastocysts derived from a cross in which 25% of the fertilized embryos were expected to have the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype often showed brown discoloration and abnormal morphology. These abnormal blastocysts likely have the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype, and the brown discoloration may be attributable to ferritin overexpression and sequestration of ferric iron in ferritin, whereas abnormal morphology may be due to concomitant functional iron deficiency. These results demonstrate that IRPs are indispensable for regulation of mammalian iron homeostasis at the post-implantation stage of murine embryonic development.

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**Keywords:** 5 total-embryogenesis; Ferritin; Iron metabolism; IRP1; IRP2

## Introduction

Iron is an important cofactor for numerous fundamental processes in cells, including heme synthesis, respiratory chain function, and DNA synthesis. In rapidly dividing cells, including those transformed by the oncogene *c-myc*, expression of iron metabolism genes alters to increase availability of iron to growing cells [1]. Rapid cell division is a feature of embryogenesis, but it is not known how embryos acquire iron at various developmental stages, particularly during early development as zygotes divide and differentiate into blasto-

cysts. The oocyte may contain sufficient iron to sustain early embryonic development, as the iron storage protein ferritin has been detected in *Xenopus* oocytes [2], and is likely present in pre-implantation embryos. In development of fertilized eggs, transferrin is not required for progression of development from the two-cell to the blastocyst stage [3], consistent with the fact that these early embryos are bathed in mucosal secretions, which usually contain lactoferrin, a member of the transferrin family commonly expressed by epithelial cells and found in bodily secretions including milk, tears, and in nasal and genital secretions [4]. Interestingly, lactoferrin is expressed in pre-implantation embryos between the 2 and 4 cell stage and through the blastocyst stage of development [5], and lactoferrin may contribute to iron acquisition in the pre-implantation embryos by binding to embryonic lactoferrin receptors [6].

After implantation of the blastocyst, the embryo develops a placenta, which provides an interface with the maternal

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circulation and indirect access of the embryo to maternal transferrin iron. Transferrin receptor 1 (TfR1) is clearly expressed by embryonic ectoderm and embryo-derived syncytiotrophoblasts in day 6 post-implantation tissues [7], suggesting that Fe-transferrin uptake plays an important role in early embryonic post-implantation development and beyond [8]. Mice homozygous for a null allele of TfR1 die before embryonic day 12.5, and manifest abnormal development of erythroid and neurologic lineages [9]. The fact that embryos developed up to embryonic day 12.5 despite complete lack of TfR1 suggests that another TfR-like protein was able to substitute for TfR1 during this time. TfR2 is a good candidate for providing TfR function during early embryogenesis in mammals [10], because according to the Cancer Genome Anatomy project, TfR2 is highly expressed in embryonic stem cells and embryonic brain and kidney (cgap.nci.nih.gov). In the model organism zebrafish, there are two TfR1 homologues and one TfR2 homologue, and embryos with an erythroid-specific TfR1a mutation develop anemia, whereas those with TfR1b mutations develop abnormalities in embryonic somatic tissues [11]. Thus, TfR-mediated iron uptake plays an important role in iron acquisition and viability in embryos that develop beyond the blastocyst stage in mice and in zebrafish.

Iron regulatory proteins 1 and 2 (IRP1 and IRP2) are cytosolic proteins that post-transcriptionally regulate expression of iron metabolism genes by binding to RNA stem-loop structures in transcripts known as iron-responsive elements (IREs) (reviewed in [12]). To characterize the contribution of each of these genes to regulation of iron metabolism, we generated mice with targeted deletions of IRP1 or IRP2. IRP1<sup>-/-</sup> animals developed no overt phenotype [13], whereas IRP2<sup>-/-</sup> mice developed adult-onset neurodegeneration, hyperferritinemia [14], microcytic anemia, and elevated red cell protoporphyrin IX levels [15]. We concluded that the major reason that IRP2 dominates iron metabolism is that IRP2 is stable and active at prevailing tissue oxygen concentrations (3–6%) and cells can compensate for loss of IRP1 activity by increasing IRP2 levels [16]. At normal tissue oxygen concentrations, the bifunctional protein IRP1 mainly functions as a cytosolic aconitase [17], and only a small percentage of IRP1 is in the IRE-binding form. In addition, IRP1 does not appear to activate its IRE-binding activity in animals maintained on a low iron diet, whereas IRP2 is very responsive to dietary iron changes [13]. Although IRP1 is not required for regulation of iron metabolism in WT animals, IRP2<sup>-/-</sup> animals that also lack one copy of IRP1 develop much more severe anemia and neurodegeneration after weaning, though they are born in normal numbers [18]. Thus, retention of one allele of IRP1 is sufficient to allow animals to develop and survive through adulthood.

Here, we demonstrate the animals that completely lack both IRPs cannot survive through gestation. Although blastocysts with IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype can be detected, they are not able to implant and further develop, thus indicating that IRP function is required for embryonic development.

## Materials and methods

### Genotyping

IRP1<sup>-/-</sup> and IRP2<sup>-/-</sup> animals were genotyped and bred as previously described [13,14,18].

### Mating

Multiple crosses between double heterozygous animals (IRP1<sup>+/-</sup> IRP2<sup>+/-</sup>) and animals that were heterozygous at one allele and homozygous at the other were performed, and pups from these matings were genotyped by Southern blots.

### Embryo dissection

To determine the stage of embryonic lethality, embryos of approximately 6.5, 9.5, 13.5, and 16.5 days were dissected and genotyped using PCR genotyping as described below.

### Blastocyst harvest

Blastocysts were harvested from IRP1<sup>-/-</sup> IRP2<sup>+/-</sup> matings as follows: superovulation was performed as previously described [19,20], animals were sacrificed, and blastocysts obtained from flushing the dissected oviducts with BMOC-3 medium were examined on a dissecting microscope (Wild M5-40553, Wild Heerbrugg, Switzerland). Abnormal blastocysts were photographed in a petri dish using the 2× objective of a Nikon Eclipse E600 microscope with a DXM1200F digital camera.

### Blastocyst genotyping

Blastocysts were isolated individually, and lysed in 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris–Cl pH 8.0, 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20, 200 µg/ml Proteinase K, and 10 mM DTT. To genotype the IRP2 allele, PCR primers for the wild-type and recombinant allele were utilized. For WT and IRP2<sup>-/-</sup> genotyping, a primer from exon 4, 5'ACGTGTCCTGTTTGCCTTGTTTC3', was used in combination with a downstream primer found in either the WT or in the recombinant allele. After 30 cycles, a WT PCR product of 570 bp was generated using the common 5' primer with the reverse complement of a sequence from exon 6, 5'CTCACAGTGGACCACTCTTTACAGA3'. A recombinant product of 627 nucleotides was generated with the reverse complement of the targeted deletion construct—5'GAATGTATGTGGAACAGAGGCTG3'.

### PCR genotyping of murine embryos

Embryos from day 6.5, day 10, and day 15 were dissected and individually lysed and genotyped [20] with generous help from Heiner Westphal and colleagues at NICHD.

## Results

Male and female animals carrying null alleles of IRP1 and IRP2 were bred, and pups were genotyped. Genotype distributions of progeny expected after breeding were calculated and compared with genotypes of offspring. As displayed in Table 1, no live pups with the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype were born and distributions strongly supported that the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype was embryonic lethal. To determine when lethality occurred, animals with 6.5, 9.5, 13.5 and 16.5 day-old embryos were dissected and individual embryos were genotyped. There were no IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> embryos detected in any post-implantation embryos.

Since we could not find IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> embryos at the earliest post-implantation stages, we harvested blastocysts after performing timed matings. We harvested approximately 200 blastocysts from different matings, and we successfully genotyped approximately 50 blastocysts, of which approximately 10 had the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype, two of which are shown by PCR genotyping in Fig. 1. Interestingly, numerous blastocysts showed brownish discoloration (Fig. 2) and abnormal morphology (not shown). Although immunohistochemistry can be performed on blastocysts [21], we were unable to successfully perform immunohistochemistry to determine ferritin or TfR content on fixed and permeabilized blastocysts. We also attempted to grow blastocysts to generate enough material to characterize ferritin and TfR content, but we were unable to grow IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> blastocysts and we therefore could not obtain enough material for molecular studies. However, the numbers of abnormal discolored blastocysts correlated roughly with the expected numbers of IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> embryos, although for technical reasons, none of the brown blastocysts were typed directly.

To assess the likelihood that functional iron deficiency may play an important role in the progression of disease in IRP2<sup>-/-</sup> mice, we fed animals on a low iron diet or a high iron diet after weaning. Animals on a low iron diet died at

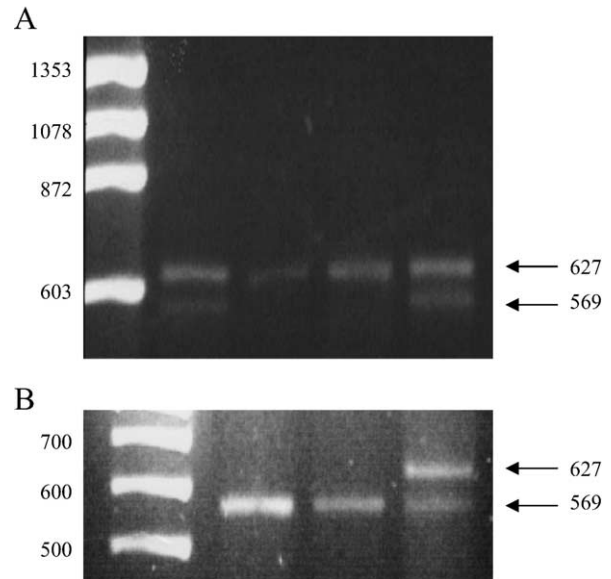


Fig. 1. Blastocysts with the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype are detectable after blastocyst harvest and PCR genotyping. Blastocysts were harvested from animals after timed matings were performed. Blastocysts were flushed from the maternal oviducts after approximately 3.5 days of gestation, analyzed for morphology, and lysed for genotyping as described in Materials and methods. Since the parents in crosses performed for blastocyst harvest were homozygous for the null allele of IRP1, only the IRP2 allele needed to be genotyped. To generate a PCR band for the null allele, a sequence from the targeting construct was used with a primer from WT exon 4. To generate the WT band, a sequence from exon 6 was used with the exon 4 primer. According to predictions, the WT band should be 569 nucleotides in length, whereas the null allele band should be 627 nucleotides in length. Representative genotyping gels are shown in panels A and B. In panel A, lanes 2 and 5 contain bands of 569 and 627 and are heterozygous. Lanes 3 and 4 contain only the 627 nucleotide band of the null allele, and these lanes therefore identify blastocysts with the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype. In panel B, WT blastocysts are genotyped in lanes 2 and 3, whereas the genotype of the blastocyst in lane 4 is IRP1<sup>-/-</sup> IRP2<sup>+/-</sup>.

16 ± 3 months, whereas those on a high iron diet lived to 26 ± 2 months, a difference that was statistically different according to the Student's *t* test at *P* < 0.0001. Notably, Wt

Table 1  
Genotypes of offspring of mating between IRP1 and IRP2 heterozygous and knockout mice<sup>a</sup>

Crossing	Total number of pups	IRP1+/+ IRP2+/+	IRP1+/+ IRP2+/-	IRP1+/- IRP2+/+	IRP1+/- IRP2+/-	IRP1-/- IRP2+/+	IRP1+/- IRP2+/-	IRP1+/- IRP2-/-	IRP1-/- IRP2+/-	IRP1-/- IRP2-/-
IRP1+/+ IRP2+/- and IRP1+/+ IRP2+/-	184	11 (11.5)	27 (23)	21 (23)	9 (11.5)	13 (11.5)	62 (46)	17 (23)	24 (23)	0 (11.5)
IRP1-/- IRP2+/- and IRP1+/+ IRP2+/-	148	0 (0)	0 (0)	24 (18.5)	0 (0)	25 (18.5)	43 (37)	17 (18.5)	39 (37)	0 (18.5)
IRP1-/- IRP2+/- and IRP1-/- IRP2+/-	157	0 (0)	0 (0)	0 (0)	0 (0)	52 (39.25)	0 (0)	0 (0)	105 (78.5)	0 (39.25)
IRP1-/- IRP2+/- and IRP1+/+ IRP2-/-	8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (2)	3 (2)	1 (2)	0 (2)
IRP1+/+ IRP2-/- and IRP1+/+ IRP2+/-	10	0 (0)	4 (1.25)	0 (1.25)	0 (0)	0 (0)	6 (2.5)	0 (2.5)	0 (1.25)	0 (1.25)
IRP1+/+ IRP2-/- and IRP1+/+ IRP2-/- (high iron diet maters only)	4	0 (0)	0 (0)	0 (0)	2 (1)	0 (0)	0 (0)	2 (2)	0 (0)	0 (1)

Genotypes of offspring of IRP1+/+ IRP2+/- matings and IRP1-/- IRP2+/- matings reveal that no IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> survive gestation.

Multiple crosses between double heterozygous animals (IRP1+/+ IRP2+/-) and animals that were heterozygous at one allele and homozygous at the other were performed, and pups from these matings were genotyped at 21 days of age. Matings in which the mother's genotype was IRP1+/+ IRP2-/- infrequently resulted in pregnancy or production of viable offspring. Similarly, fathers with the IRP1+/+ IRP2-/- genotype were difficult to breed. However, when animals were maintained on a high iron diet, viable offspring were produced, although they never had the IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> genotype. Genotypes of breeding mice are shown in the left-hand panel, and genotypes of pups are shown in vertical columns. In each case, the number of pups born with each genotype is included, and the expected number of pups with that genotype based upon the expected genotype distribution according to Mendelian genetics from a given cross is shown below in parentheses. Despite analyzing numerous offspring, no IRP1<sup>-/-</sup> IRP2<sup>-/-</sup> pups were born.

<sup>a</sup> The values in parenthesis indicate the expected numbers.



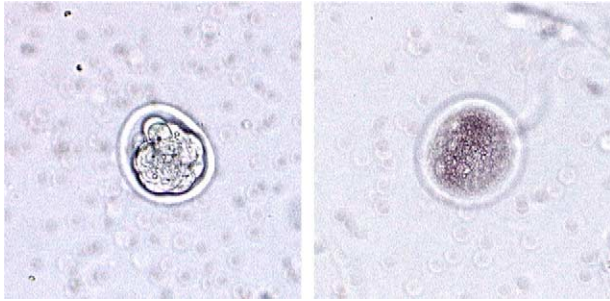


Fig. 2. Brown blastocysts with abnormal morphologies are common in crosses that produce  $IRP1^{-/-} IRP2^{-/-}$  blastocysts. Each time blastocysts were harvested from matings that produced  $IRP1^{-/-} IRP2^{-/-}$  blastocysts, up to 25% of the blastocysts were abnormal in appearance. On some occasions, blastocysts were not round, and on many occasions, blastocysts were visibly brown as shown.

animal survival did not change significantly on the low vs. the high diet, with both groups averaging survival to approximately 24 months. In addition, breedings between  $IRP^{+/-} IRP2^{-/-}$  animals never produced progeny until these animals were placed on a high diet (see Table 1).

## Discussion

Our studies establish that  $IRP1^{-/-} IRP2^{-/-}$  embryos can develop to the blastocyst stage of development but cannot progress past the stage of implantation. Our previous studies of  $IRP2^{-/-}$  animals and  $IRP1^{+/-} IRP2^{-/-}$  animals revealed that these animals have an iron-insufficiency anemia, which is largely attributable to lack of TfR1 and uptake of diferric transferrin in developing erythroid cells, but which is also exacerbated by sequestration of iron in excess ferritin [15]. The anemia of  $IRP1^{+/-} IRP2^{-/-}$  animals is significantly worse than that of  $IRP1^{+/+} IRP2^{-/-}$  animals, consistent with the observation that these animals have less TfR1 and more ferritin, because loss of one  $IRP1$  allele further compromises the ability of  $IRP2^{-/-}$  animals to protect TfR1 mRNA from degradation and to prevent ferritin translation in erythroid precursors [15] and in the other tissues, including the central nervous system [18]. Thus, it would be logical to predict that animals that completely lacked functional IRPs would have a profound loss of TfR1 and marked overexpression of ferritin. We suggest that overexpression of ferritin and sequestration of ferric iron may account for the brownish blastocyst discoloration, which we saw in blastocysts only when  $IRP1^{-/-} IRP2^{-/-}$  blastocysts were among the genotypes present. We hypothesize that the fertilized oocyte contains enough stored iron to support multiple early cell divisions, but that when the embryo implants and cells became dependent on transferrin iron uptake, inability to protect the TfR transcript from degradation, together with ferritin overexpression and concomitant sequestration of iron that should be used in the metabolism of growing cells, and possibly misregulation of other targets such as ferroportin [22], results in functional iron deficiency and early embryo death. It is likely that multiple abnormalities account for the embryogenesis failure, because when loss of TfR1 expression is the sole

abnormality, embryos progress much further in development, to day 12.5 approximately [9].

The fact that loss of one copy of  $IRP1$  (to create  $IRP1^{+/-} IRP2^{-/-}$  mice) exacerbated the neurodegeneration phenotype of the  $IRP2^{-/-}$  mice strongly implies that neurodegeneration is a direct consequence of loss of  $IRP$  activity. It has been suggested that the neurodegeneration of  $IRP2^{-/-}$  mice may be an off-target effect of the genetic engineering [23], even though these authors acknowledge that their  $IRP2$  deletion is incomplete and animals were phenotyped at less than 1 year of age, prior to the onset of significant neurodegenerative disease in the  $IRP2^{-/-}$  animals [24]. We suggest that other iron metabolism abnormalities related to loss of IRPs, particularly ferritin overexpression and concomitant functional iron insufficiency, compromise embryonic development. The notion that functional iron deficiency is an important consequence of loss of  $IRP$  function is supported by the discovery that  $IRP2^{-/-}$  mice die much earlier on a low iron diet vs. a high iron diet, and fertility of  $IRP1^{+/-} IRP2^{-/-}$  mice increases on a high iron diet.

Our results demonstrate that function of the  $IRP$  regulatory system is indispensable even at early stages of embryonic development, and they underscore the importance of post-transcriptional regulation of iron metabolism not only in adult animals, but also throughout embryonic development.

## Acknowledgments

We thank William F. Rall, Mahua Mukhopadhyay, Heiner Westphal and Chris Ward of NIH for help with dissection of blastocysts and embryos.

## References

- [1] K.J. Wu, A. Polack, R. Dalla-Favera, Coordinated regulation of iron-controlling genes, H-ferritin and  $IRP2$ , by c-MYC, *Science* 283 (1999) 676–679.
- [2] S. Durand, P. Abadie, S. Angeletti, S. Genti-Raimondi, Identification of multiple differentially expressed messenger RNAs in normal and pathological trophoblast, *Placenta* 24 (2003) 209–218.
- [3] D.K. Gardner, M. Lane, Alleviation of the ‘2-cell block’ and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters, *Hum. Reprod.* 11 (1996) 2703–2712.
- [4] P.P. Ward, O.M. Conneely, Lactoferrin: role in iron homeostasis and host defense against microbial infection, *Biometals* 17 (2004) 203–208.
- [5] P.P. Ward, M. Mendoza-Meneses, B. Mulac-Jericevic, et al., Restricted spatiotemporal expression of lactoferrin during murine embryonic development, *Endocrinology* 140 (1999) 1852–1860.
- [6] Y.A. Suzuki, B. Lonnerdal, Baculovirus expression of mouse lactoferrin receptor and tissue distribution in the mouse, *BioMetals* 17 (2004) 301–309.
- [7] B.L. Drake, J.R. Head, Transferrin receptor expression in early postimplantation mouse trophoblast and associated tissues, *Placenta* 11 (1990) 535–547.
- [8] R. Fuchs, I. Ellinger, Endocytic and transcytotic processes in villous syncytiotrophoblast: role in nutrient transport to the human fetus, *Traffic* 5 (2004) 725–738.
- [9] J.E. Levy, O. Jin, Y. Fujiwara, F. Kuo, N.C. Andrews, Transferrin receptor is necessary for development of erythrocytes and the nervous system, *Nat. Genet.* 21 (1999) 396–399.
- [10] H. Kawabata, R. Yang, T. Hirama, et al., Molecular cloning of transferrin

- receptor 2. A new member of the transferrin receptor-like family, *J. Biol. Chem.* 274 (1999) 20826–20832.
- [11] R.A. Wingert, A. Brownlie, J.L. Galloway, et al., The chianti zebrafish mutant provides a model for erythroid-specific disruption of transferrin receptor 1, *Development* 131 (2004) 6225–6235.
- [12] K. Pantopoulos, Iron metabolism and the IRE/IRP regulatory system: an update, *Ann. N. Y. Acad. Sci.* 1012 (2004) 1–13.
- [13] E.G. Meyron-Holtz, M.C. Ghosh, K. Iwai, et al., Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis, *EMBO J.* 23 (2004) 386–395.
- [14] T. LaVaute, S. Smith, S. Cooperman, et al., Targeted deletion of iron regulatory protein 2 causes misregulation of iron metabolism and neurodegenerative disease in mice, *Nat. Genet.* 27 (2001) 209–214.
- [15] S.S. Cooperman, E.G. Meyron-Holtz, H. Olivier-Wilson, M.C. Ghosh, J.P. McConnell, T.A. Rouault, Microcytic anemia, erythropoietic protoporphyria, and neurodegeneration in mice with targeted deletion of iron-regulatory protein 2, *Blood* 106 (2005) 1084–1091.
- [16] E.G. Meyron-Holtz, M.C. Ghosh, T.A. Rouault, Mammalian tissue oxygen levels modulate iron-regulatory protein activities in vivo, *Science* 306 (2004) 2087–2090.
- [17] T. Rouault, R. Klausner, Regulation of iron metabolism in eukaryotes, *Curr. Top. Cell. Regul.* 35 (1997) 1–19.
- [18] S.R. Smith, S. Cooperman, T. Lavaute, et al., Severity of neurodegeneration correlates with compromise of iron metabolism in mice with iron regulatory protein deficiencies, *Ann. N. Y. Acad. Sci.* 1012 (2004) 65–83.
- [19] M. Legge, M.H. Sellens, Optimization of superovulation in the reproductively mature mouse, *J. Assist. Reprod. Genet.* 11 (1994) 312–318.
- [20] A. Nagy, M. Gertsenstein, R. Kristina Vintersten Behringer (Eds.), *Recovery and In Vitro Culture of Pre-Implantation-Stage Embryos*, Cold Spring Harbor Laboratory Press, 2003, pp. 201–203.
- [21] M. Mohan, J.R. Malayer, R.D. Geisert, G.L. Morgan, Expression of retinol-binding protein messenger RNA and retinoic acid receptors in preattachment bovine embryos, *Mol. Reprod. Dev.* 60 (2001) 289–296.
- [22] S. Abboud, D.J. Haile, A novel mammalian iron-regulated protein involved in intracellular iron metabolism, *J. Biol. Chem.* 275 (2000) 19906–19912.
- [23] B. Galy, D. Ferring, B. Minana, et al., Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2), *Blood* 106 (2005) 2580–2589.
- [24] B. Galy, D. Ferring, M. Benesova, V. Benes, M.W. Hentze, Targeted mutagenesis of the murine IRP1 and IRP2 genes reveals context-dependent RNA processing differences in vivo, *RNA* 10 (2004) 1019–1025.